Appendix A

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Are all nitrosamines concerning? A review of mutagenicity and carcinogenicity data

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ABSTRACT

The control of potentially mutagenic impurities in pharmaceutical products is of key importance in assessing carcinogenic risk to humans. The recent discovery of nitrosamine impurities in several marketed pharmaceuticals has increased interest in their mutagenic and carcinogenic potential. This chemical class is considered part of a 'cohort of concern', indicating that standard control protocols, such as the use of a threshold of toxicological concern (TTC), cannot be applied. Whilst some nitrosamines are known to be exceptionally potent carcinogens, it's not clear whether this is a property of all members of the class. To investigate the mutagenic and carcinogenic potential of nitrosamines, data was extracted from published literature to augment that already present in the Vitic and Lhasa Carcinogenicity Databases. This data was analysed to assess the application of the ICH M7 guideline to nitrosamine impurities, with respect to the predictivity of the Ames test for carcinogenic potential and the distribution of carcinogenic potency. It was found that 18% of nitrosamines were considered non-carcinogenic. Nitrosamines showed a greater correlation between mutagenicity and carcinogenicity compared to non-nitrosamine compounds. Whilst nitrosamines, in general, are more potent carcinogens than non-nitrosamines, there is a significant overlap between the two distributions of TD50s for each class.

1. Introduction

Mutagenic impurities are compounds which may react directly with DNA and potentially introduce genetic mutations, even at low concentrations, which can initiate tumour formation. The assessment and control of mutagenic impurities in pharmaceutical products with respect to their carcinogenic risk is outlined in the ICH M7 guideline. This guideline describes how theoretically acceptable levels of human exposure can be derived for mutagenic impurities lacking adequate experimental carcinogenicity data. For example, the threshold of toxicological concern (TTC) is a commonly used acceptable intake level derived by linear extrapolation from preclinical TD_{50} values (Cheeseman et al., 1999) (the dose at which the probability of remaining tumour-free after chronic administration for the standard lifespan would be halved), which are themselves derived from experimental tumour incidence data. In the context of ICH M7, the TTC-based approach applies a threshold of 1.5 μg/day as the level at which a compound with inadequate experimental carcinogenicity data can be considered as having a negligible carcinogenic risk in humans. Where adequate data is available, acceptable intakes can be derived on a compound- or class-specific basis (Bercu et al., 2018). In the absence of mutagenicity data for an impurity, the assessment of mutagenic potential may be made using *in silico* predictions from two complementary (quantitative) structural activity relationship ((Q)SAR) methodologies, one expert rule-based and one statistical-based (ICH, 2017).[1]

N-Nitrosamines (nitrosamines) [Fig. 1] are a class of compounds which humans are commonly exposed to in small doses via tobacco and food products (for example, cured meats). The recent discovery of nitrosamine impurities in several marketed pharmaceuticals has led to increased interest in their mutagenic and carcinogenic potential. Regulatory authorities have requested that marketing authorisation holders review the manufacturing process and finished products for all

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[1] Abbreviations: CPDB- Carcinogenic Potency Database; EMA- European Medicines Agency; LCDB- Lhasa Carcinogenicity Database; NDEA- N-nitrosodiethylamine; NDMA- N-nitrosodimethylamine; NPV- negative predictive value; NTP- National Toxicology Program; OECD- Organisation for Economic Cooperation and Development; PPV- positive predictive value; (Q)SAR- (quantitative) structural activity relationship; TTC- threshold of toxicological concern.

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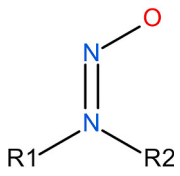

Fig. 1. Substructure for N-nitrosamine compounds.

synthesised active pharmaceutical ingredients to identify the potential for the presence of nitrosamine impurities. This is to be done using a risk-based approach to prioritise evaluations and subsequent testing (EMA, 2019a; Health Canada, 2019; Swissmedic, 2019). Should a risk be identified, then confirmatory testing and a change to the manufacturing process would be required to remove the risk. The ICH M7 guideline includes nitrosamines in a 'cohort of concern', alongside potent carcinogens such as aflatoxin-like and alkyl-azoxy compounds. This means that acceptable levels of exposure are likely to be significantly lower than the TTC defined by the guideline. In this sense, the 1.5 μg/day threshold cannot be applied, and the presence of a nitrosamine must be controlled on a case-by-case basis using carcinogenicity data for closely related compounds.

Whilst some nitrosamine compounds are exceptionally potent carcinogens, it is unclear whether this is a universal property of all members of this class. The European Medicines Agency have set interim acceptable limits for selected nitrosamine impurities to 26.5 ng/day or 96 ng/day, based on their similarity to N-nitrosodiethylamine (NDEA) or N-nitrosodimethylamine (NDMA), respectively (EMA, 2019b). The current work was undertaken to investigate how the mutagenic activity of nitrosamines is indicative of their carcinogenic potential and the degree to which tumourigenic potency varies across this chemical class. This may then inform the how theoretically safe levels of human exposure could vary depending on the structure of individual nitrosamine compounds. With regard to mutagenicity data, analysis focussed on the bacterial reverse mutation assay (Ames test), which is commonly used as an *in vitro* predictor of a compound's *in vivo* carcinogenic potential (ICH, 2017).

2. Method

2.1. Mutagenicity and carcinogenicity data

Vitic is a commercially available, structure-searchable database which contains curated toxicology data collected predominantly from public sources (Lhasa Limited, 2020). To expand the coverage of nitrosamines in Vitic, a series of literature searches were carried out using PubMed (U.S. National Library of Medicine (NLM)) (to provide a list of articles containing Ames test and/or rodent carcinogenicity data relating to nitrosamines. Publications containing nitrosamines that were absent from Vitic were prioritised for data extraction, with a view to expanding the chemical coverage of nitrosamines in the database.

Following data collection and its addition to the Vitic database, all Ames test and rodent carcinogenicity data contained within Vitic was reviewed. To compare the mutagenicity and carcinogenicity data for each compound, overall Ames and rodent carcinogenicity calls were derived following a previously defined workflow (Thresher, 2016) with minor modifications. This process consisted of the following steps:

1. Mutagenicity data was selected from the Vitic database where the test type was labelled as either 'Ames test' or 'bacterial reverse mutation assay' and the test species was given as either *Salmonella typhimurium* or *Escherichia coli*. Carcinogenicity data was selected from studies using rodent species.
2. Studies with a Klimisch score of 3 ('Not reliable') were removed from the data set.
3. Data records were grouped into subsets of similar studies. For Ames data this was done according to the bacterial strain, metabolic activation system and study protocol used (e.g. plate incorporation). For carcinogenicity data, subsets were defined according to the species and sex of the test subjects, the route of administration and the duration of exposure to the test substance.
4. For each subset, an activity call was generated based on the original author's call for each study. Subsets containing a mixture of positive and negative, or equivocal and negative studies were given an activity call of 'conflicted'.
5. Ames subsets were prioritised by preferentially selecting those containing well defined strain and metabolic activation information. Subsets where these values were not specified were only used to determine the overall call where no complete subsets where present. No prioritisation was carried out with the carcinogenicity subsets.
6. The most conservative subset call was taken as the overall compound call.

These calls were then used to examine the concurrence of mutagenic and carcinogenic activity for nitrosamines, both as a class and juxtaposed with the compounds in Vitic which do not contain the nitrosamine toxicophore (non-nitrosamines). As such, compounds resolving as either conflicted or equivocal were removed from the data set.

2.2. Carcinogenic potency

The Carcinogenic Potency Project (CPDB), project, originally created by Gold et al. (1984), was a database of long-term carcinogenicity study data gathered from public literature and the U.S. National Toxicology Program (NTP) and included calculated TD_{50} values. The data in the CPDB was last updated in 2007, and so the Lhasa Limited Carcinogenicity Database, (LCDB) (Lhasa Limited) was created to safeguard the CPDB data. The freely available LCDB contains both the original CPDB TD_{50} values together with Lhasa-generated TD_{50} values created using a script based on the original CPDB methodology (Thresher et al., 2019). This data was examined in relation to nitrosamines, both to clarify the correlation between CPDB- and Lhasa-generated values specifically for this class of compounds and to examine how the tumourigenic potency distribution compares to the remaining non-nitrosamine carcinogens.

3. Results

3.1. Assessment of mutagenicity and carcinogenicity data

The Vitic database (Vitic, 2020.1) now contains a total of 479 nitrosamines: 381 with Ames test data and 228 with rodent carcinogenicity data, of which 178 of these contain both Ames test and rodent carcinogenicity data. The increase in data compared to the previous version of the Vitic database (Vitic, 2018.1) is shown in Fig. 2. The number of nitrosamines in the database increased by 24%, with a 35% increase in those with Ames test data, 55% increase with rodent carcinogenicity data and 105% increase in those with both Ames test and rodent carcinogenicity data. In addition to novel nitrosamines, Ames and carcinogenicity data for 150 nitrosamines already in Vitic was expanded to include broader information on the range of strains and study protocols.

In total, Ames activity calls were generated for 381 nitrosamines and 14,193 non-nitrosamines, with carcinogenicity calls generated for 228 nitrosamines and 2913 non-nitrosamines. In all four data sets the number of conflicted or equivocal calls was negligible (Fig. 3). The highest number of conflicted and equivocal calls was 5% in the non-nitrosamine Ames calls and 3% in the non-nitrosamine carcinogenicity calls, respectively.

The nitrosamines showed an almost identical proportion of positive and negative calls in the Ames and carcinogenicity studies. A greater divergence was observed in the non-nitrosamines, with a higher

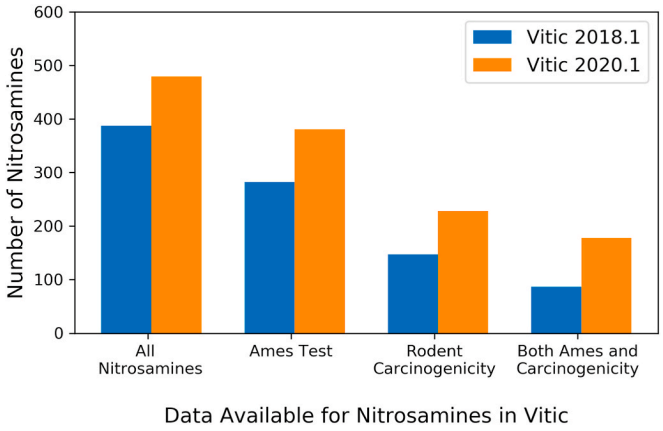

Fig. 2. Number of nitrosamines with Ames and/or rodent carcinogenicity data in Vitic 2018.1 and 2020.1.

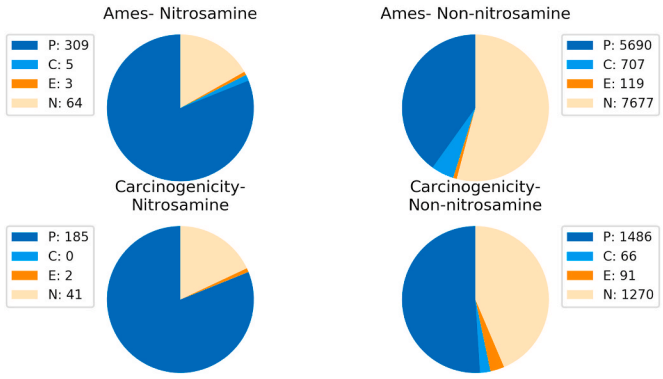

Fig. 3. Proportion of Ames and carcinogenicity activity calls for the nitrosamines and non-nitrosamines in Vitic 2020.1. P= Positive; C= Conflicted; E = Equivocal; N= Negative.

proportion being carcinogenic than mutagenic. While it is unsurprising that a much higher proportion of nitrosamines are mutagenic or carcinogenic compared to non-nitrosamines, given the presence of nitrosamines in the 'cohort of concern', it is surprising is that approximately 18% of nitrosamines appear to be non-carcinogenic.

3.2. Predictivity of Ames test for carcinogenic activity

After removal of compounds with either a conflicted or equivocal call, a total of 171 nitrosamines and 1862 non-nitrosamines containing both Ames and carcinogenicity calls remained. For each compound, these calls were compared to give an indication of the predictivity of the Ames test for carcinogenicity [Supplementary Table 1, Fig. 4].

The nitrosamines show a high balanced accuracy, sensitivity, positive predictive value (PPV) and negative predictive value (NPV), indicating that the Ames test can predict the carcinogenic potential of nitrosamines. Indeed, there was a greater correlation between the Ames and carcinogenicity calls than for non-nitrosamine compounds. The high specificity and comparatively low sensitivity of the nitrosamines (compared to the non-nitrosamines) could indicate lack of negative predictivity by the Ames test for this class. However, the number of non-carcinogenic nitrosamines is too low to make a definitive conclusion.

The underlying reason for the high number of "false negatives" in the non-nitrosamine data is unclear; however, the possibility that several of these are either non-genotoxic carcinogens or active through a genotoxic mechanism not detected by the Ames test is consistent with the higher proportion of carcinogens compared to mutagens in this data set.

In the nitrosamines data set, 8 compounds were described as "false negatives", i.e. they had a negative Ames call and positive carcinogenicity call. A review of the study information available for these compounds shows that only 2 have been tested in 5 bacterial strains, both in the presence and absence of a metabolic activation system, as described in the current Organisation for Economic Co-operation and Development (OECD, 1997) test guideline 471 [Supplementary Table 2]. This indicates that the remaining studies may not offer a robust assessment of the mutagenic potential of these nitrosamines and further testing under OECD guideline 471 compliant conditions would be advisable to confirm them as non-mutagens.

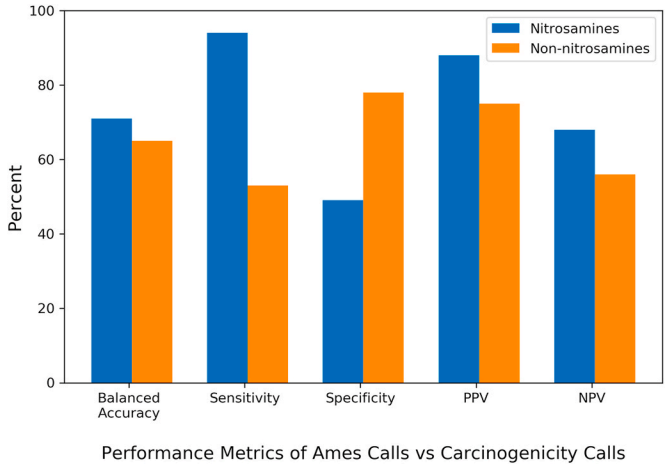

Fig. 4. Performance metrics for the comparison of Ames and carcinogenicity calls derived from the Vitic 2020.1 data.

Within the nitrosamine Ames test data set approximately 34% of the subsets defined during step 3 of the summary workflow did not conform to the current OECD 471 guideline. However, the removal of these subsets from the summary workflow resulted in only a small decrease in the total number of nitrosamines with summary Ames calls (8%), as many compounds contain data from multiple subsets. Of the remaining nitrosamines, the absence of the non-OECD compliant subsets did not significantly alter the summary calls generated, remaining identical in 92% of cases. Regarding the predictive performance of the Ames test for carcinogenic potential, removal of the non-OECD compliant Ames studies had a minimal impact on the correlation between Ames test and carcinogenicity summary calls. This resulted in a balanced accuracy of 68.9, compared to 69.4 when including the non-OECD compliant Ames data for the same compounds.

3.3. Evaluation of carcinogenic potency

The LCDB contains a total of 137 nitrosamines, of which 117 were considered carcinogenic by the original study authors. Of these, 46 contain both Lhasa and CPDB TD_{50} values. Previous work has demonstrated a high correlation between the original CPDB TD_{50} values and the Lhasa-calculated TD_{50} values (Thresher et al., 2019). The distribution of log Lhasa TD_{50} and log CPDB TD_{50} values for both nitrosamines and non-nitrosamines within the LCDB reiterates the high correlation between the CPDB- and Lhasa-calculated values, especially for the nitrosamines.

Nitrosamines are typically more potent carcinogens compared to the non-nitrosamine carcinogens in this data set, in that the log Lhasa TD_{50} values are generally lower for these compounds. While there is substantial overlap in potency between the two data sets, the mean nitrosamine log Lhasa TD_{50} value (−0.433) is considerably lower than that of the non-nitrosamines (1.418) [Fig. 5].

NDEA is the most potent nitrosamine for which carcinogenicity data is available. Although it is commonly used to illustrate the carcinogenic potential of nitrosamines, NDEA is exceptionally potent compared to

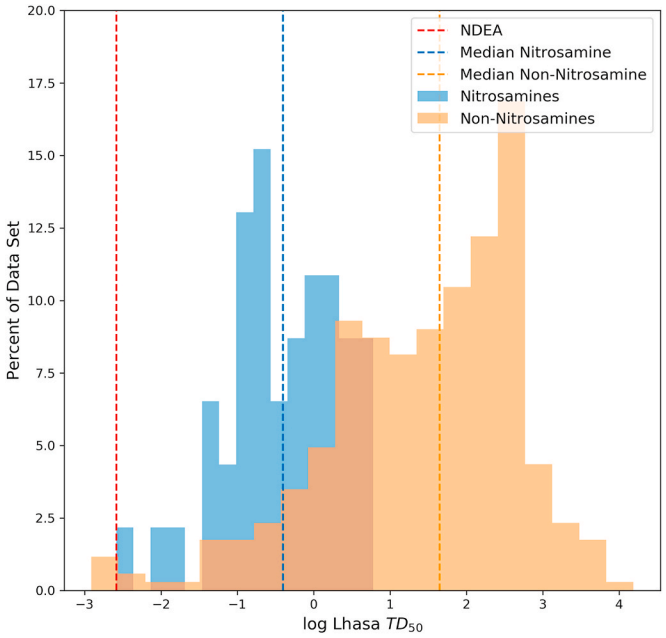

Fig. 5. Distribution of log Lhasa TD50 values for nitrosamine and non-nitrosamine compounds as a proportion of the respective data sets within the LCDB.

most other nitrosamines [Fig. 5]. The log Lhasa TD_{50} value for NDEA (−2.585) is considerably lower than the class mean (−0.433), with only N-nitroso-N-methyl-2-phenylethylamine (−2.10) displaying a similar value. The potency of NDEA is more comparable to aflatoxin B1 (−2.458), in the non-nitrosamine data set, which is also present in the 'cohort of concern'.

4. Discussion

A considerable amount of mutagenicity and carcinogenicity data for nitrosamines has been added to that already available in Vitic and the LCDB. This has shown that, despite the majority of nitrosamines being carcinogenic, approximately 18% of the data set resolved as non-carcinogenic based on the expanded data in Vitic. However, consideration should be given to assessing the study protocol used, specifically regarding the duration of exposure and highest tested doses, when regarding the validity of study results. Comparison of the activity calls generated from the Vitic data showed a strong correlation between the Ames and carcinogenicity results. This correlation proved to be greater than that observed for the non-nitrosamine compounds in Vitic, indicating that the Ames test is highly predictive of carcinogenic potential for this chemical class. The presence of 'false negative' Ames test results in the data set may be due to a lack of studies covering an adequate range of bacterial strains. While the standard assay conditions throughout industry often consist of the use of rat S9 under a plate incorporation protocol, the use of hamster S9 and/or a preincubation protocol is consistent with the current OECD 471 guideline. Removing non-OECD compliant subsets from the Ames summary call workflow did not significantly alter the final calls generated. Therefore, the use of the ICH M7 guideline in assigning an impurities classification based on adequate mutagenicity data is appropriate for this class of compounds.

Nitrosamines are specifically listed in ICH M7 within the 'cohort of concern' along with other highly potent carcinogens, such as aflatoxins, as they are considered to pose a significant risk to human health at doses below the standard acceptable intakes defined in the guideline. Instead, an assessment of the carcinogenic risk must be made for nitrosamines on a case-by-case basis. In response to the discovery of nitrosamine impurities in marketed pharmaceuticals, the EMA has imposed interim acceptable limits of 26.5 ng/day for selected nitrosamines based on their similarity to NDEA (EMA, 2019b). Although carcinogenic nitrosamines, as a class, are typically more potent than other carcinogens, they exhibit a wide distribution of log TD_{50} values, from NDEA at −2.585 to 1-nitrosopiperazine at 0.781. This distribution overlaps with that of the non-nitrosamine carcinogens, including some not present in the 'cohort of concern'. The mean log Lhasa TD_{50} value of −0.433 suggests NDEA may not be an exemplar of the carcinogenic potency of this chemical class.

5. Conclusion

The available data shows that not all nitrosamine compounds are concerning. The high correlation between the Vitic Ames test and carcinogenicity data shows that the Ames test, as described in the OECD 471 guideline, can be used as an indicator of carcinogenic potential for nitrosamines. While carcinogenic nitrosamines display a more potent TD_{50} distribution when compared to non-nitrosamine carcinogens in the LCDB, this potency does vary within the class. Further analysis of the structures of nitrosamines is required to determine what common features influence carcinogenic potency.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2020.104749.

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N-NITROSO COMPOUNDS AND HUMAN CANCER

A MOLECULAR EPIDEMIOLOGIC APPROACH

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Choi, B. C. K. (Faculty of Medicine, U. of Toronto, Toronto, Ontario, Canada M5S 1A1). *N*-Nitroso compounds and human cancer: a molecular epidemiologic approach. *Am J Epidemiol* 1985;121:737–43.

***N*-Nitroso compounds are known to be potent animal carcinogens. However, evidence of their effect on human cancers is inconclusive, and further investigations are needed. A mathematical model to create indices of nitrate, nitrite, and *N*-nitrosamine exposures, both exogenous and endogenous, is described in this paper. Estimation of the endogenous formation is based on the current knowledge of biochemistry and chemical kinetics of these compounds. The model can be applied to analyze data regarding the dietary history and use of tobacco products and alcoholic beverages obtained from epidemiologic questionnaires. Exposure levels of study and control subjects to these compounds can then be compared by conventional epidemiologic methods. This is an approach toward the combination of conventional epidemiologic methods and laboratory findings to study disease etiology.**

cancer; molecular models; nitroso compounds

Carcinogenicity of *N*-nitrosamines was first discovered by Magee and Barnes (1). Since then, *N*-nitroso compounds have been extensively studied in many laboratories. It is now well established that *N*-nitroso compounds are potent carcinogens in laboratory animals including mammals, birds, fish, and amphibia (2). The target organs of the action of various *N*-nitroso compounds in animal studies have been found to include liver, esophagus, stomach, nasal cavity, pharynx, kidney, lung, bronchus, brain, spinal cord, tongue, intestine, bladder, skin, ovary, uterus, mammary gland, vagina, testis, lymph node, blood vessel, thymus, and pancreas (2–5).

In view of the large number of different animal species affected, it is highly unlikely that man is an exception. Suggestions of associations of *N*-nitroso compounds with human cancers including brain tumors, esophageal, and gastric cancers have been obtained from epidemiologic studies of exposures to substances containing high levels of preformed *N*-nitroso compounds, as in a recent case-control study by Preston-Martin et al. (6), and from results of epidemiologic studies that high consumptions of nitrate (7) and nitrite (8–10) are related to human cancers. Evidence from studies of nitrates and nitrites is indirect and relies on the fact that nitrite, mainly derived from ingested nitrates, may react in vivo with ingested nitrogen compounds to form *N*-nitroso compounds (7). Thus far, the evidence regarding *N*-nitroso compounds and human cancer is inconclusive, and further investigations are needed. The main difficulty is that no satisfactory index of exposure to nitrites and *N*-nitroso compounds

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is available. Current carcinogenesis theories indicate that endogenous production of nitrite and *N*-nitroso compounds is usually a more important source of human exposure than exogenous intake (2), but conventional epidemiologic techniques tend to ignore this important fact.

Epidemiology has now reached a stage in which conventional epidemiologic methods may combine with laboratory findings to give a new approach to the study of disease etiology. This is the "molecular epidemiologic" approach (11). This paper describes a mathematical model to create indices of nitrate, nitrite, and *N*-nitrosamine exposures, based on the current knowledge of biochemistry and chemical kinetics regarding these compounds. Both exogenous intake and endogenous formation of these compounds are estimated by the model. The mathematical model is suitable for the analysis of data regarding the dietary history and the use of tobacco products and alcoholic beverages obtained from epidemiologic questionnaires.

An example using a real epidemiologic study is given at the end of this paper to illustrate the application of the model.

THE MATHEMATICAL MODEL

In the mathematical model, concentrations of nitrate and nitrite are expressed in terms of the two ions (NO_3^- and NO_2^-). Nitrate and nitrite are measured in milligrams (mg) per day, and *N*-nitroso compounds in micrograms (μg) per day.

Difficulties in analytic techniques have thus far limited research of *N*-nitroso compounds mainly to the analysis of volatile *N*-nitrosamines (2). Nitrosodimethylamine (NDMA) is at present the most extensively studied *N*-nitroso compound in food. Information concerning other *N*-nitroso compounds in food is virtually nonexistent. In this model, therefore, NDMA is taken to be a representative compound for all *N*-nitroso compounds. A high NDMA exposure is assumed to be correlated with a high total *N*-nitroso compound exposure. We have made an assumption that NDMA is representative of all *N*-nitroso compound exposure, but until further information regarding other *N*-nitroso compounds in food is available, exposure to NDMA is the best index of exposure to all *N*-nitroso compounds.

Nitrate exposure

Exogenous nitrate intake. Human exposure to nitrate is mainly exogenous from food and water (12). Major sources of exogenous nitrate include vegetables (estimated per capita daily intake of 86.1 mg), cured meats (9.4 mg), bread (2.0 mg), fruits and fruit juices (1.4 mg), water (0.7 mg), and milk and milk products (0.2 mg) (2). Vegetables constitute the single major source of nitrate, accounting for about 86 per cent of the daily intake.

Nitrate contents of many food items have been quantified (12, chapter 5). From an epidemiologic questionnaire recording the starting date, ending date, frequency and amount of consumption of each type of food, the total nitrate intake in mg/day (EXONITRATE) from various dietary sources can be estimated.

Endogenous nitrate formation. There were reports that nitrate might be endogenously synthesized (13, 14), but the evidence is inconclusive. Moreover, the amount of endogenous nitrate formation has never been quantified (15). Endogenous nitrate formation is therefore not taken into account in the mathematical model.

Consequently, total nitrate exposure in mg/day (TOTNITRATE) is assumed to be the same as the exogenous intake:

$$\text{TOTNITRATE} = \text{EXONITRATE} \quad (1)$$

Nitrite exposure

Exogenous nitrite intake. Human exposure to nitrite may be exogenous from food and water and endogenous from the reduction of ingested nitrate (16). Major sources of exogenous nitrite include cured meats (estimated per capita daily intake of 2.38 mg), vegetables (0.20 mg), and bread (0.02

mg) (2). Cured meats and vegetables together account for about 99 per cent of the daily intake.

Nitrite contents of many food items have been quantified (12, chapter 5). From the epidemiologic questionnaire, the total nitrite intake in mg/day (EXONITRITE) can be estimated.

Endogenous nitrite formation. An important source of nitrite exposure in humans is the endogenous formation from ingested nitrate. This endogenous reduction of nitrate to nitrite is a first-order reaction (12, p. 4-4):

$$NO_3^- \rightarrow NO_2^- + O$$

Since 1 mol of nitrate gives 1 mol of nitrite, the rate of nitrite formation is proportional to the nitrate concentration, that is,

$$\text{Rate} = k[NO_3^-].$$

Formation of nitrite may occur in the saliva, stomach, and intestine (2, 15, 16). According to Spiegelhalder et al. (17), approximately 25 per cent of ingested nitrate is recirculated into the saliva, and approximately 20 per cent of salivary nitrate is reduced to nitrite. In other words, approximately 5 per cent of ingested nitrate is converted to nitrite in the saliva. Formation of nitrite in the stomach and intestines is still controversial, and the amount has not been quantified at present (2, 15). The best available estimate is that approximately 5 per cent of the ingested nitrate is reduced to nitrite in the human body. This amount of endogenous nitrite is added to the amount of ingested nitrite to give the total nitrite exposure in mg/day (TOTNITRITE):

$$\text{TOTNITRITE} = \text{EXONITRITE} + 0.05*\text{TOTNITRATE}. \quad (2)$$

N-Nitroso compound exposure

Human exposure to *N*-nitroso compounds may result exogenously from ingestion or inhalation of preformed compounds in the environment, and endogenously from nitrosation of amino precursors in the body (18).

Exogenous nitrosodimethylamine (NDMA) intake. Major sources of exogenous *N*-nitroso compounds are cigarettes (estimated per capita daily intake of 17 μg), beer (1 μg), car interiors (0.5 μg), cosmetics (0.4 μg), cured meat (0.2 μg), and whisky (0.03 μg) (12, table 7-17).

NDMA contents of meat products (19, 20), tobacco smoke (12, chapter 7), and alcoholic beverages (21) have been quantified. From the epidemiologic questionnaire, the total NDMA intake in μg/day (EXONITROSO) from these sources can be estimated. The intake from car interiors and cosmetics is difficult to determine at this stage, and is not considered in the model.

Endogenous NDMA formation. Endogenous formation of *N*-nitroso compounds are variable, depending upon the dietary intake of nitrate and nitrite (e.g., people on an average US diet have a per capita daily production of 1.3 μg; vegetarians have a production of 12 μg) (12, table 8-4).

The endogenous production of nitrosamines from the nitrosation of secondary amines by nitrite is believed to involve the following steps (2, 22):

$$\underset{\text{(nitrite)}}{NO_2^-} + H^+ \rightarrow \underset{\text{(nitrous acid)}}{HNO_2}, \quad \text{(step 1)}$$

$$2HNO_2 \rightleftharpoons H_2O + \underset{\text{(dinitrogen trioxide)}}{N_2O_3}, \quad \text{(step 2)}$$

and

$$\underset{\text{(amine)}}{R_2NH} + N_2O_3 \rightarrow \underset{\text{(nitrosamine)}}{R_2NNO} + HNO_2. \quad \text{(step 3)}$$

Since 2 mol of nitrous acid are required to produce 1 mol of N_2O_3 (step 2), which reacts with 1 mol of amine to give 1 mol of nitrosamine (step 3), the rate of nitrosation is proportional to the amine concentration and the square of nitrite concentration (2), that is,

$$\text{Rate} = k[R_2NH][NO_2^-]^2.$$

Since the concentration of amine in the human body is very high compared with that of nitrite, this reaction is determined by the concentration of only one reactant, namely nitrite. Thus, the reaction of amine with nitrite is second order in nitrite, i.e., proportional to the square of the nitrite concentration. This implies that if the nitrite concentration is high, the formation of nitrosamines will occur to a much greater extent.

Under normal human physiologic conditions, in which daily amine intake is assumed to be 4,000 mg, the equation for formation of nitrosoproline (NPRO) in a stomach with a 900-ml capacity has been quantified (12, p. 8-28) to be

$$[\mu g \text{ NPRO}] = 0.04865 [\text{mg } NO_2^-]^2.$$

Since the nitrosation rate of dimethylamine is about 22 times slower than that of proline (23), the conversion constant for dimethylamine is 0.0022, so that

$$[\mu g \text{ NDMA}] = 0.0022 [\text{mg } NO_2^-]^2.$$

Total NDMA exposure in μg/day (TOTNITROSO), which is the sum of exogenous intake and endogenous production, is estimated by

$$\text{TOTNITROSO} = \text{EXONITROSO} + 0.0022(\text{EXONITRITE} + 0.05*\text{TOTNITRATE})^2. \quad (3)$$

An example

In a case-control study of adult brain tumors conducted in southern Ontario between 1979 and 1983 which used 210 newly diagnosed cases and 210 matched hospital controls, subjects were administered an epidemiologic questionnaire by an interviewer. The questionnaire recorded the type, starting year, ending year, frequency (per day, week, month, or year), and amount (in grams) of consumption of meat products, other food items, tobacco products, and beverages.

Exogenous nitrate and nitrite intakes were calculated as the total intake in mg/day from various food items (using table 1) and meat products (using table 2). Exogenous NDMA intake was calculated as the

TABLE 1

*Nitrate and nitrite contents of selected food items**

	Nitrate (mg/kg)	Nitrite (mg/kg)
Fresh meats	10	1
Fruits	20	0
Fruit juices	2.0 (mg/liter)	0
Baked goods and cereals	12	2.6
Milk and milk products	0.5 (mg/liter)	0
Water	1.3 (mg/liter)	0
Vegetables		
Artichoke	12	0.4
Asparagus	44	0.6
Beans		
Green	340	0.6
Lima	54	1.1
Dry	13	NR†
Beets	2,400	4
Broccoli	740	1
Brussels sprouts	120	1
Cabbage	520	0.5
Carrots	200	0.8
Cauliflower	480	1.1
Celery	2,300	0.5
Corn	45	2
Cucumber	110	0.5
Eggplant	270	0.5
Endive	1,300	0.5
Kale/collard	800	1.0
Leek	510	NR
Lettuce	1,700	0.4
Melon	360	NR
Mushroom	160	0.5
Okra	38	0.7
Onion	170	0.7
Parsley	1,010	NR
Peas	28	0.6
Pepper, sweet	120	0.4
Potatoes		
White	110	0.6
Sweet	46	0.7
Pumpkin and squash	400	0.5
Radish	1,900	0.2
Rhubarb	2,100	NR
Spinach	1,800	2.5
Tomatoes	58	NR
Turnip	390	NR
Turnip greens	6,600	2.3

* Source: Committee on Nitrite and Alternative Curing Agents in Food (12, tables 5-3, 5-8, and p. 5-25).

† NR, not reported.

TABLE 2

*Nitrate, nitrite, and nitrosodimethylamine (NDMA) contents of selected meat products**

	No. of samples	Nitrate (mg/kg)	Nitrite (mg/kg)	NDMA (μg/kg)
Unsmoked side bacon	9	134	12	2
Unsmoked back bacon	2	160	8	0
Peameal bacon	2	16	21	5
Smoked bacon	13	52	7	2
Other bacon	7	58	48	1
Corned beef	11	141	19	0
Cured corn beef	3	852	9	4
Corned beef brisket	7	90	3	2
Pickled beef	4	70	23	2
Canned corned beef	3	77	24	2
Ham	12	105	17	1
Smoked ham	6	138	50	0
Cured ham	2	767	35	3
Cooked ham	9	109	17	0
Canned ham	1	44	5	0
Cottage roll	4	553	28	0
Semicured ham	6	73	23	1
Unsmoked sausage	2	21	7	0
Smoked sausage	6	129	12	0
Wiener	13	97	7	2
Beef weiner	3	109	7	1
Other sausage	13	20	7	2
Luncheon meat	12	42	5	1
Pickle and pimiento loaf	3	51	4	0
Meat, macaroni, and cheese loaf	4	75	22	1
Mock chicken loaf	6	107	11	3
Other luncheon meats	4	87	13	1
Salami	12	86	12	1
Beef salami	4	71	27	2
Bologna	20	77	19	2
Belitalia (garlic)	1	247	5	0
Pepperoni (beer)	2	149	23	0
Summer sausage	1	135	7	0
Ukrainian sausage (Polish)	6	77	15	3
German sausage	1	71	17	0

* Values are means computed from results of Panalaks et al. (19, 20), and have been converted from concentrations of the sodium salt to concentrations of the ion.

total intake in μg/day from meat products (using table 2) and from tobacco products and alcoholic beverages (using table 3).

Total nitrite exposure was calculated as the sum of exogenous nitrite intake and endogenous nitrite production (using equation 2). Similarly, total NDMA exposure was calculated as the sum of exogenous NDMA intake and endogenous NDMA production (using equation 3).

For 210 control subjects, the mean per capita daily intakes for these compounds are (mean ± standard error)

Exogenous nitrate = 44.31 ± 4.04 mg/day;
Exogenous nitrite = 0.50 ± 0.05 mg/day;
Exogenous NDMA = 1.14 ± 0.25 μg/day;
Total nitrite = 2.71 ± 0.34 mg/day;
Total NDMA = 1.21 ± 0.25 μg/day.

These results are consistent with estimates from previous reports. For example, four estimates of exogenous nitrate intake range from 39–100 mg/day (12, table 5-18), and four estimates of exogenous nitrite intake range from 0.34–2.6 mg/day (12, table 5-19). Previous theoretic estimates for en-

TABLE 3
Nitrosodimethylamine (NDMA) contents of tobacco products and alcoholic beverages

	NDMA
Plain cigarette*	0.02 μg/cigarette
Filter cigarette*	0.0065 μg/cigarette
Cigar*	NR†
Beer‡	2.5 μg/liter
Spirits§	0.5 μg/liter
Wine¶	0.05 μg/liter

* Source: Committee on Nitrite and Alternative Curing Agents in Food (12, tables 7-5, 7-17).
† NR, not reported.
‡ Average of 215 samples from Spiegelhalder et al. (24).
§ Average of 176 samples from Walker et al. (21).
¶ Average of 29 samples from Walker et al. (21).

dogenous nitrite formation range from 3.5 mg/day (12, table 8-1) to 8.62 mg/day (25), and our results indicate that approximately 2.21 mg of nitrite are formed daily from the reduction of ingested nitrate. No previous estimate for exogenous and endogenous NDMA daily exposure is available for comparison with our results.

Among these 210 control subjects, therefore, the exposure to nitrite is approximately 18 per cent exogenous and 82 per cent endogenous, and the exposure to NDMA is approximately 94 per cent exogenous and 6 per cent endogenous.

DISCUSSION

The mathematical model described above can be used to estimate both the exogenous intake and the endogenous formation of nitrate, nitrite, and NDMA. This approach represents one of the first steps toward the combination of conventional epidemiologic methods and laboratory findings to study disease etiology. Exposure levels (exogenous, endogenous, or total) of study and control subjects to these compounds can then be compared by conventional epidemiologic methods. Calculation of total exposures, as a sum of exogenous intake and endogenous formation, is new and requires the knowledge of the biochemistry and chemical kinetics of the molecules involved. Thus, the mathematical model presented here may have to be revised constantly, when new information is available. Improved quantitation methods will give more accurate figures of nitrate, nitrite, and *N*-nitroso compound contents of food and other substances. Further development in laboratory work will provide more information regarding endogenous nitrate formation, exogenous exposure to *N*-nitroso compounds other than NDMA, exposure to *N*-nitroso compounds from car interiors and cosmetics, and direct estimation of the velocity constant for formation of NDMA without having to use an analogy to the velocity constant of *N*-nitrosoproline. In addition, when quantitative data are available, the effects of nitrosation inhibitors, such as ascorbic acid and alpha-tocopherol, should be considered. The present model does not take into account the variation of the metabolism and of the chemical kinetics of nitrate, nitrite, and *N*-nitroso compounds among individuals that is due to differences in physiology, age, and general health. This should be accounted for in future models as well.

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